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Ancestry of American Polyploid *Hordeum* Species with the I Genome Inferred from 5S and 18S–25S rDNA

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• *Background and Aims* The genus *Hordeum* exists at three ploidy levels (2*x*, 4*x* and 6*x*) and presents excellent material for investigating the patterns of polyploid evolution in plants. Here the aim was to clarify the ancestry of American polyploid species with the I genome.
• *Methods* Chromosomal locations of 5S and 18S–25S ribosomal RNA genes were determined by fluorescence *in situ* hybridization (FISH). In both polyploid and diploid species, variation in 18S–25S rDNA repeated sequences was analysed by the RFLP technique.
• *Key Results* Six American tetraploid species were divided into two types that differed in the number of rDNA sites and RFLP profiles. Four hexaploid species were similar in number and location of both types of rDNA sites, but the RFLP profiles of 18S–25S rDNA revealed one species, *H. arizonicum*, with a different ancestry.
• *Conclusions* Five American perennial tetraploid species appear to be alloploids having the genomes of an Asian diploid *H. roshevitzii* and an American diploid species. The North American annual tetraploid *H. depressum* is probably a segmental alloploid combining the two closely related genomes of American diploid species. A hexaploid species, *H. arizonicum*, involves a diploid species, *H. pusillum*, in its ancestry; both species share the annual growth habit and are distributed in North America. Polymorphisms of rDNA sites detected by FISH and RFLP analyses provide useful information to infer the phylogenetic relationships of I-genome *Hordeum* species because of their highly conserved nature during polyploid evolution.

Key words: Chromosome, FISH, genome, *Hordeum*, *in situ* hybridization, karyotype evolution, phylogeny, RFLP, ribosomal genes, wild barley.

INTRODUCTION

The genus *Hordeum* (Triticeae) is classified into 31 species (45 taxa in total, including subspecies and cytotypes) and 51 cytotypes exist at the diploid, tetraploid and hexaploid levels with a basic chromosome number of $x = 7$ (Baden and von Bothmer, 1994; von Bothmer *et al*., 1995). Phylogenetic relationships of the species in this genus have been studied intensively. Characters examined include morphology, metaphase I (MI) chromosome pairing in interspecific hybrids (von Bothmer *et al*., 1986, 1987, 1988), C-banding patterns and morphology of satellited (SAT) chromosomes (Linde-Laursen *et al*., 1995), isozymes (Jørgensen, 1986), repetitive DNA sequences (Molnar *et al*., 1989; Svitashev *et al*., 1994), nuclear and chloroplast DNA sequences (Doebley *et al*., 1992; Komatsuda *et al*., 1999; Nishikawa *et al*., 2002; El-Rabey *et al*., 2002; Petersen and Seberg, 2003), and chromosomal distribution of cloned repetitive DNA sequences (de Bustos *et al*., 1996; Taketa *et al*., 1999*a*, *b*, 2001). Analysis of MI chromosome pairing (von Bothmer *et al*., 1995) revealed the presence of four basic genomes (H, I, X and Y). Other studies have confirmed the division of the genus into four groups corresponding to the above four basic genomes, but detailed species relationships, especially those within a group, have not been fully resolved owing to the complicated evolution. A recent molecular cytogenetic study (Taketa *et al*., 1999*a*) revealed that three polyploid species/cytotypes, *H. secalinum*, *H. capense* and *H. brachyantherum* ssp. *brachyantherum* 6*x*, were allopolyploids having combinations of the X and I genomes. Thus, the genus is classified into five genome groups, namely H, I, X, Y and XI (Taketa *et al*., 1999*a*). In this paper, genome designation follows that of Taketa *et al*. (2001), namely, *H. vulgare* and *H. bulbosum* both carry the H genome, *H. marinum* carries the X genome, *H. murinum* has the Y genome, and the remaining species share variants of the I genome. In the present genome nomenclature, the genome symbols H and I have been swapped relative to the designation by von Bothmer *et al*. (1995). This is because the symbol H was allocated to the genomes of *H. vulgare* and *H. bulbosum* at the 7th International Barley Genetics Symposium (Linde-Laursen *et al*., 1997).

The I-genome group is the largest of the five groups and includes 25 species. Fourteen species are diploid, five are tetraploid and four are hexaploid; the remaining two species exists at two and three ploidy levels. The I-genome species are distributed from Central Asia to the American Continents (von Bothmer *et al*., 1995). The I-genome group contains many morphologically similar species, and the nature of polyploidy and species relationships of this group remain largely uncertain despite the many studies cited above.

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Fluorescence *in situ* hybridization (FISH) is a powerful molecular cytogenetic technique to reveal genome constitution and species' relationships in plants (Heslop-Harrison, 2000). In the genus *Hordeum*, the technique has been applied to restricted species (de Bustos *et al*., 1996; Taketa *et al*., 1999*b*, 2001). Previously we analysed the physical location of two types of ribosomal DNA (5S rDNA and 18S–5.8S–25S rDNA, hereafter referred to as 18S–25S rDNA) in 15 of the 16 I-genome diploid species (Taketa *et al*., 1999*b*, 2001). These studies demonstrated the usefulness of rDNA sites as chromosome landmarks for investigating karyotype evolution and phylogeny in the genus *Hordeum*. Similar approaches have been utilized successfully in several other plant genera and species (*Aegilops*, Bedaeva *et al*., 1996; *Gossypium*, Hanson *et al*., 1996; *Brachyscome linearloba*, Adachi *et al*., 1997; *Hypochaeris*, Cerbah *et al*., 1998; *Trifolium*, Ansari *et al*., 1999; *Arachis*, Raina and Mukai, 1999; *Sanguisorba*, Mishima *et al*., 2002). In the present study, research was extended to nine I-genome polyploid species of American origin, providing new information on their origin. Moreover, the study was complemented by Southern hybridization analyses with the wheat 18S–25S rDNA probe pTa71, to detect rDNA sequence variation.

MATERIALS AND METHODS

Plant materials

Five tetraploid and four hexaploid species of the I-genome group, which are distributed in the American Continents, were analysed by FISH and Southern hybridization (Table 1). A South American I-genome diploid species *H. comosum*, which was not available in our previous study, was also included. For reference, 18 taxa (in total 21 accessions, Table 1) were selected for Southern hybridization analyses with the 18S–25S rDNA probe. Detailed information on collection sites of the materials is given in the Appendix. Seeds are preserved at both Kagawa University, Japan and the Nordic Gene Bank, Sweden, and are available upon request from the senior author.

Fluorescence in situ *hybridization*

Actively growing root-tips from germinating seeds or hydrocultured plants were treated in ice water at 0 °C for 16–24 h to accumulate metaphases and fixed with 3 : 1 (v/v) 100 % ethanol : acetic acid. Root tips were stained with a 2 % acetocarmine solution and squashed in a drop of 45 % acetic acid according to the acetocarmine squash technique (see Fukui, 1996). Two DNA clones, pTa794 and pTa71 were used as probes. Clone pTa794 is a *Bam*HI fragment of the 5S rDNA, having a 120-bp coding sequence for the 5S rRNA gene and the intergenic spacers isolated from common wheat, *Triticum aestivum* (Gerlach and Dyer, 1980). For clone pTa71, the 18S–25S rDNA is a 9-kb *Eco*RI fragment from common wheat, containing the coding sequences for the 18S, 5·8S and 25S rRNA genes and the intergenic spacer sequences (Gerlach and Bedbrook, 1979). The *in situ* hybridization procedure described by Taketa *et al*. (1999*b*) was adopted. Slides were examined with an Olympus BX-50 epifluorescence microscope with appropriate filter sets (U-MWU for UV, U-MWIB for FITC, U-DM-Cy3 for Cy3 and U-DM-DA/FI/TX for simultaneous visualization of all fluorochromes). Photographs were taken on Fujicolor Super HG400 color print film. Photographs were scanned and processed in Adobe Photoshop using only cropping and processing functions that affect all pixels in the image equally. The number of rDNA sites was determined from observation of at least five metaphases with clear signals.

rDNA–RFLP

For Southern analysis, the total genomic DNA (0·5 μg) of two North American tetraploid species, *H. depressum* H2089 and *H. jubatum* H1922 were digested with eight restriction enzymes, *Bam*HI, *Bgl*II, *Dra*I, *Eco*RI, *Eco*RV, *Hin*dIII, *Xba*I and *Xho*I separately, electrophoresed in 0·8 % agarose gels, and transferred to a positively charged nylon membrane. The blot was hybridized with the purified rDNA probe from the clone pTa71 according to a standard protocol of ECL chemiluminescence (Amersham). Hybridization and washing stringencies were 78 % and 86 %, respectively. Hybridization sites were detected using ECL detection reagents and recorded directly on Hyperfilm-ECL (Amersham). From this preliminary survey, *Eco*RV was selected as the most informative restriction enzyme for detecting RFLP in wild *Hordeum* and used in the subsequent analysis. Exposure times for luminographs (shown in Fig. 3) ranged from 15–60 min.

RESULTS

Chromosomal in situ *mapping of the ribosomal genes*

First, a South American diploid, *H. comosum*, was analysed. This species was found to be 'chilense type' (Taketa *et al*., 2001), having two SAT chromosome pairs (one submetacentric with double 5S rDNA sites and one metacentric), typical of American I-genome diploid species (data not shown). Figure 1A–H shows the somatic metaphase chromosomes of eight I-genome polyploid species after *in situ* hybridization with 5S and 18S–25S rDNA probes and counterstaining with 4′, 6-diamidino-2-phenylindole (DAPI). Idiograms of the haploid complement of the chromosomes with rDNA sites in all nine species are presented in Fig. 2. The present *in situ* hybridization experiment detected 5S and major and minor 18S–25S rDNA sites and achieved detection sensitivity similar to that of our previous studies (Taketa *et al*., 1999*b*, 2001). In this paper, we define SAT chromosomes as chromosomes with a major 18S–25S rDNA site because secondary constrictions were hard to observe in polyploids.

Tetraploid species

In American tetraploid species, the number of chromosome pairs with rDNA sites was five for *H. jubatum* (Fig. 1A), *H. guatemalense* (Fig. 1B), *H. fuegianum* and *H. tetraploidum* (Fig. 1D), but was four for *H. depressum* (Fig. 1C). The former four species show a perennial growth

TABLE 1. Hordeum *species analysed in this study and the rDNA fragments obtained from* Eco*RV digestion of total genomic DNA*

Species	Accession	Ploidy	Collection site	FISH image in Fig. 1	*Eco*RV digestion (kb)				Lane number in Fig. 3
					12–10	9·5–7·5	4–3	2·5	
Species used for FISH analysis									
H. depressum	H2089	4*x*	USA	C		7·5		2·5	1
	H2005	4*x*	USA	Not shown		7·5		2·5	2
H. jubatum	H1922	4*x*	USA	A	11·7, 11·5	9		2·5	3
	H1976	4*x*	USA	Not shown	11·7, 11·5	9		2·5	4
H. guatemalense	H2299	4*x*	Guatemala	B	11·5	9		2·5	6
H. fuegianum	H1418	4*x*	Argentina	Not shown	11·5	8·2, 8		2·5	7
H. tetraploidum	H6198	4*x*	Argentina	D	–	–	–	–	–
H. arizonicum	H3254	6*x*	USA	E	11·7, 11·5	9, 8·7	4, 3·2	2·5	18
H. lechleri	H6104	6*x*	Argentina	F	11·7	9, 8·5		2·5	19
H. parodii	H6294	6*x*	Argentina	G	11·5	9, 7·5		2·5	20
H. procerum	H1789	6*x*	Argentina	H	11·7	9, 8·5		2·5	21
H. comosum	H10608	2*x*	Argentina	Not shown	–	–	–	–	–
Species only used for RFLP analysis									
H. brevisubulatum ssp. *violaceum*	H315	2*x*	Iran	*	11				8
H. bogdanii	H4014	2*x*	Pakistan	*	10				9
H. roshevitzii	H9152	2*x*	China	*	11·5				10
H. brachyantherum ssp. *californicum*	H3317	2*x*	USA	*		7·5		2·5	11
	H1954	2*x*	USA	*		9		2·5	12
	H2419	2*x*	USA	–		9, 8·5		2·5	31
ssp. *brachyantherum*	JIC line 2	4*x*	unknown	**	11				5
	H2420	4*x*	USA	–	11·7	9		2·5	17
H. pusillum	H722	2*x*	USA	*		8·7	4, 3·5	2·5	13
H. intercedens	H2310	2*x*	USA	*		8·5		2·5	14
H. chilense	JIC line 1	2*x*	unknown	**		8·5		2·5	15
H. muticum	H6479	2*x*	Argentina	*		9		2·5	16
H. cordobense	H6429	2*x*	Argentina	*		8·5		2·5	22
H. flexuosum	H1116	2*x*	Argentina	*		8		2·5	23
H. patagonicum									
ssp. *musterisii*	H1358	2*x*	Argentina	*		7·5		2·5	24
ssp. *patagonicum*	H6052	2*x*	Argentina	*		9		2·5	25
ssp. *setifolium*	H1366	2*x*	Argentina	*		7·5		2·5	26
ssp. *santacrucense*	H1353	2*x*	Argentina	*		7·5		2·5	27
H. pubiflorum									
ssp. *halophilum*	H1348	2*x*	Argentina	*		9·5, 7·5		2·5	28
ssp. *pubiflorum*	H1296	2*x*	Argentina	*		9		2·5	29
H. stenostachys	H1108	2*x*	Argentina	*		8		2·5	30

* FISH images were reported in Taketa *et al*. (2001);
** reported in Taketa *et al*. (1999*b*).
–, Not analysed.

habit, but the latter is an annual species of the western USA (von Bothmer *et al*., 1995). The four perennial species, *H. jubatum* (North America and also in eastern Siberia), *H. guatemalense* (Central America) and *H. fuegianum* and *H. tetraploidum* (both from South America), had essentially the same rDNA pattern as that previously reported for the tetraploid cytotype of *H. bracyantherum* ssp. *brachyantherum* (Taketa *et al*., 1999*b*). *Hordeum bracyantherum* ssp. *brachyantherum* is a perennial from North America and Kamchatka (hereafter called *H. brachyantherum* 4*x*). In the consensus FISH pattern of perennial tetraploid species, rDNA sites are located on three pairs of submetacentric chromosomes and two pairs of metacentric chromosomes. Minor differences in the number and intensity of rDNA sites were found among the species. Except for *H. guatemalense*, all perennial tetraploid species had double 5S rDNA sites on the short arm of a submetacentric chromosome pair. Similar double 5S rDNA sites were observed in the 'chilense-type' American diploid species (Taketa *et al*., 1999*b*, 2001). We will refer to the FISH pattern found in the five American perennial tetraploid species as the 'jubatum type'.

Both accessions of *H. depressum* (H2089 and H2005) had three pairs of 5S rDNA sites and four (two major and two minor) pairs of 18S–25S rDNA sites on four pairs of chromosomes (two submetacentrics and two metacentrics). Compared to the 'jubatum type', *H. depressum* lacked a submetacentric chromosome pair with a major 18S–25S rDNA site in its short arm, but other rDNA-carrying chromosome pairs in this species can find their possible counterparts in the 'jubatum type' (Fig. 2). We designate the unique FISH pattern of *H. depressum* the 'depressum type'. Linde-Laursen *et al*. (1995) pointed out that the unique submetacentric SAT chromosome pair that is absent in *H. depressum* but is present in other American polyploid species was derived from the Asian diploid I-genome species, *H. roshevitzii*. In this paper, we will call it the 'sr SAT chromosome'.

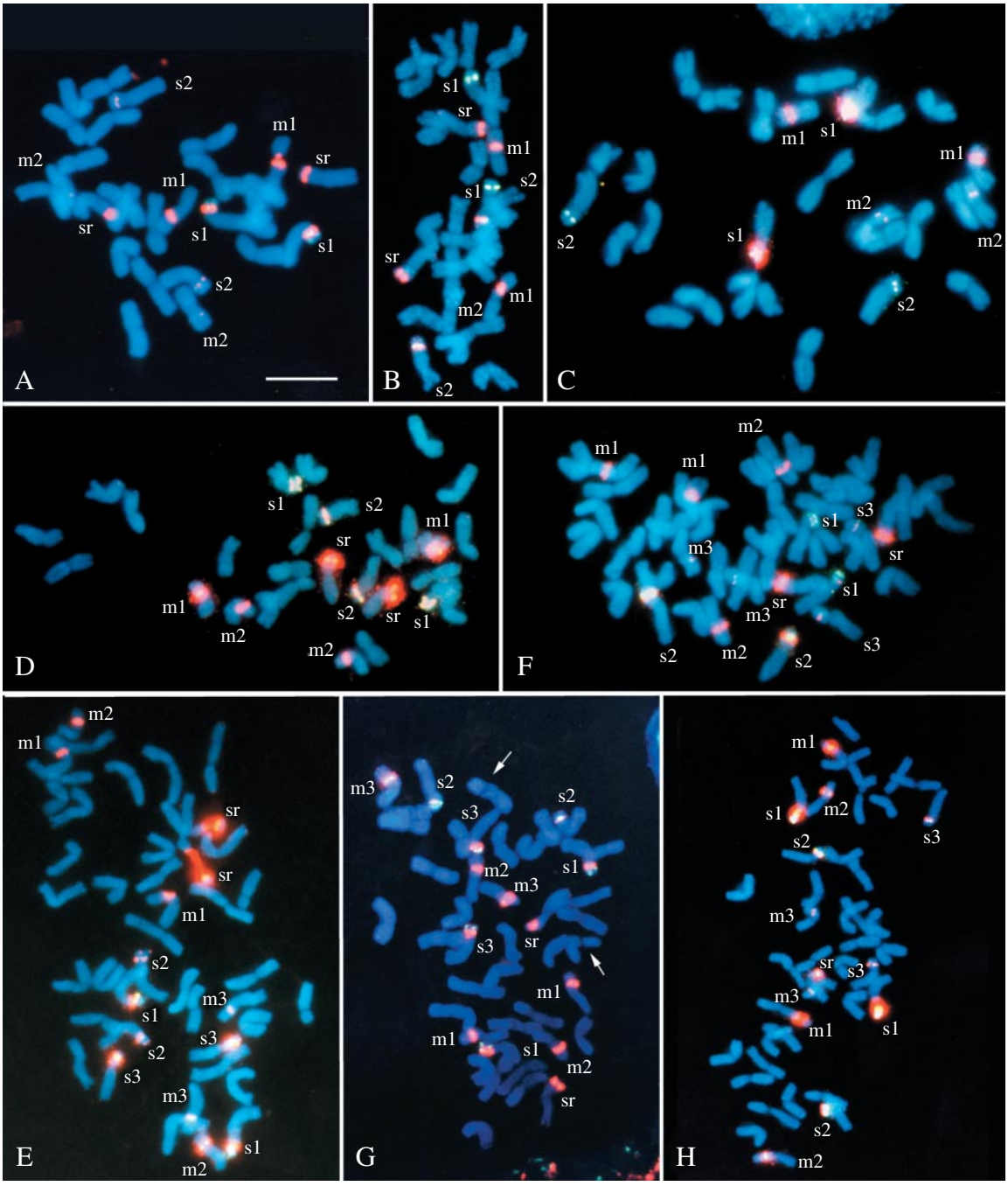

FIG. 1. Double-target *in situ* hybridization to root-tip metaphase cells in polyploid *Hordeum* species with the I genome. The micrographs were taken with a triple band filter allowing the simultaneous visualization of the DAPI-stained chromosomes (blue), the hybridization sites of the 5S rDNA (green) and the 18S–25S rDNA (red). (A) *H. jubatum* H1922; (B) *H. guatemalense* H2299; (C) *H. depressum* H2089; (D) *H. tetraploidum* H6198; (E) *H. arizonicum* H3254; (F) *H. lechleri* H6104; (G) *H. parodii* H6294; (H) *H. procerum* H1789. Chromosomes with rDNA site(s) are labelled: the first letter indicates the morphology of chromosomes, where 's' is a submetacentric and 'm' is a metacentric. The chromosome marked with 'sr' indicates the *H. roshevitzii*-specific submetacentric SAT chromosome ('sr SAT chromosome'). In (B) one m2 chromosome can not be identified due to overlapping of chromosomes. In (G) arrows indicate a tertiary constriction. The metaphase plate shown in (H) is incomplete ($2n = 41$) and one 'sr SAT chromosome' is missing. The scale bar represents 10 μm in (A) and (C); 12 μm in (B), (D–F); and 16 μm in (G) and (H).

Hexaploid species

All four American hexaploid species had rDNA sites on seven pairs of chromosomes and showed more-or-less similar FISH patterns (Figs. 1E–H, 2). The seven pairs of chromosomes with rDNA sites consisted of four submetacentrics and three metacentrics. The four submetacentric pairs included three pairs with both 5S and 18S–25S rDNA sites in their short arms and the 'sr SAT chromosome' pair. The three metacentric pairs had an 18S–25S

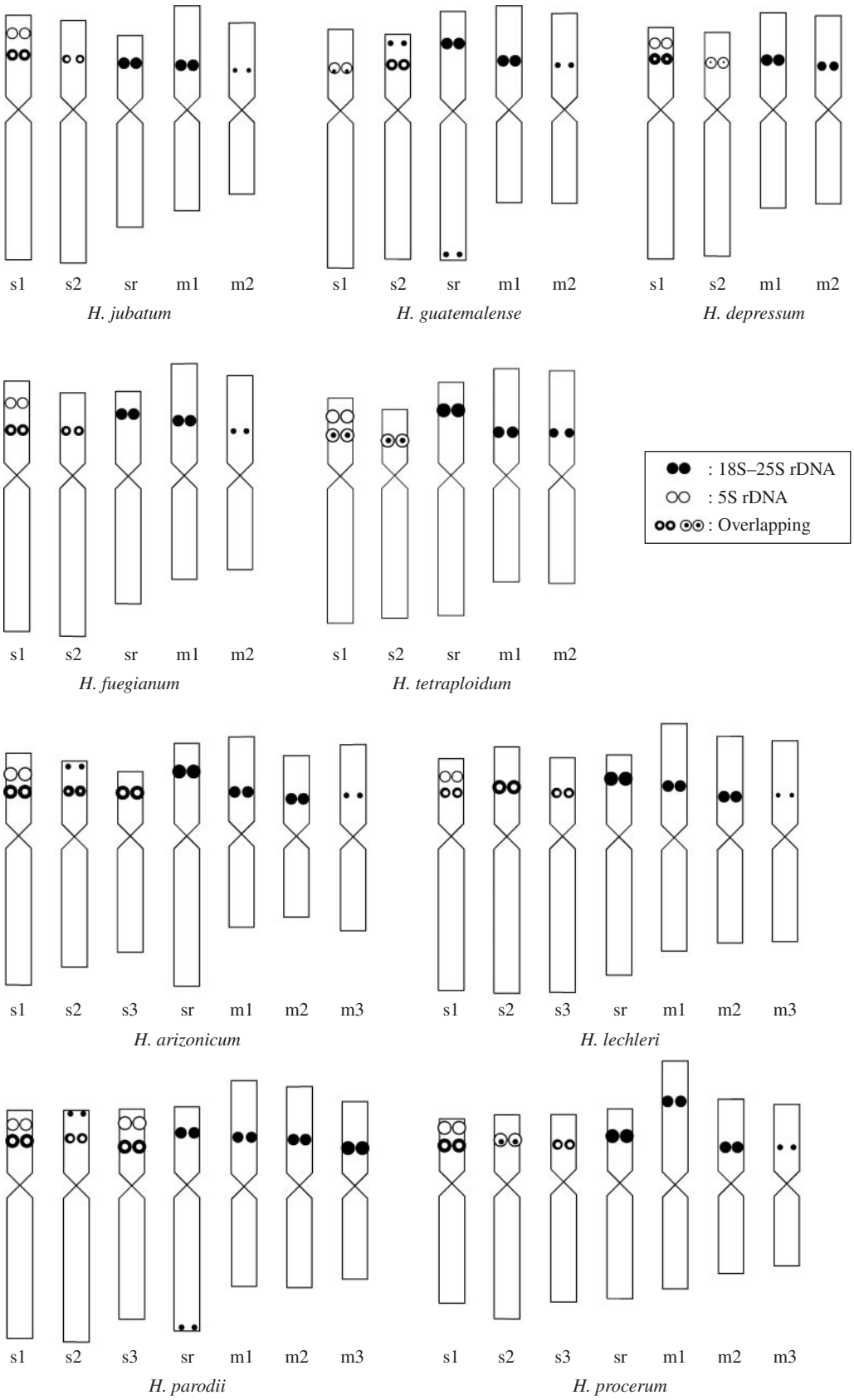

FIG. 2. Idiograms of the haploid complement of the chromosomes carrying 5S and 18S–25S rDNA in nine polyploid *Hordeum* species with the I genome investigated in the study. Letters under the chromosomes are the same as in Fig. 1.

rDNA site in one arm. Of the seven pairs of chromosomes with rDNA sites in hexaploid species, five can find their possible counterparts in the 'jubatum-type' tetraploid species, and the remaining two pairs were one submetacentric and one metacentric. These results indicate that American hexaploid species were formed from hybridization between a 'jubatum-type' tetraploid species and an American diploid species.

There was variation in the number and intensity of 5S and 18S–25S rDNA sites among hexaploid species. A North American annual/biannual species *H. arizonicum* (Fig. 1E) and a South American perennial species *H. lechleri* (Fig. 1F) were similar in FISH pattern, except that the former had an extra minor 18S–25S rDNA site in the short arm of one submetacentric SAT chromosome pair (marked s2). A South American perennial species *H. parodii* (Fig. 1G) was unique in having two pairs of submetacentric chromosomes with double 5S rDNA sites (marked s1 and s3), while the three other hexaploid species had only one pair of similar chromosomes. In addition, *H. parodii* had an extra minor 18S–25S rDNA site in the long arm terminal of the 'sr SAT chromosome' pair, and also had a metacentric chromosome pair with a tertiary constriction. In *H. procerum* (Fig. 1H), one metacentric SAT chromosome pair showed an extended chromosome arm with a more distally located 18S–25S rDNA site (marked m1), as reported by Linde-Laursen *et al*. (1990). A similar metacentric SAT chromosome pair was reported in the South American diploid *H. cordobense* (Linde-Laursen *et al*., 1989). Because the present study analysed only one accession in each hexaploid species, it is not known whether these variations are species-specific or not.

rDNA–RFLP

Two representative tetraploid species, *H. depressum* and *H. jubatum*, were analyzed by Southern hybridization using eight restriction enzymes and the pTa71 clone as a probe (Fig. 3A). Two restriction enzymes (*Hin*dIII and *Xho*I) gave no clear pattern in either species, indicating the absence of restriction sites in the pTa71 repeat unit. The other five restriction enzymes detected one or more solid fragments and detected a clear RFLP between them. *Eco*RI and *Xba*I had a single restriction site in both species and detected a pTa71 repeat unit length polymorphism. *Dra*I detected the same polymorphism, but a weak high-molecular-weight fragment was observed only in *H. depressum*. The other three enzymes, *Bgl*II, *Bam*HI and *Eco*RV, produced two or more fragments in both species and detected RFLP. In the *Bgl*II and *Eco*RV profiles, smaller fragments sum up to the repeat unit length of the respective species. The *Bam*HI profiles showed only a minor difference between the two species, and their overall patterns were similar to those reported by Molnar *et al*. (1989). Available data from different restriction enzymes suggest the presence of at least two kinds of pTa71 repeat unit sequences in each species. On the basis of these results, we selected *Eco*RV for subsequent analyses. This restriction enzyme was not used in previous rDNA–RFLP analyses of the genus *Hordeum* (Molnar and Fedak, 1989; Molnar *et al*., 1989, 1992).

In the present study, except for *H. tetraploidum*, all American I-genome polyploid species were analysed by Southern hybridization of *Eco*RV digests using the pTa71 probe. Figure 3B shows RFLP profiles and Table 1 summarizes the results. In tetraploid species, most species/accessions shared similar profiles consisting of three classes of fragments: 12–10 kb, 9·5–7·5 kb and 2·5 kb (lanes 3–7 and 17), but both accessions of *H. depressum* (lanes 1 and 2) and a *H. brachyantherum* 4*x* accession JIC line 2 (lane 5) showed a unique profile. *Hordeum depressum* had only two fragments: 7·5 kb and 2·5 kb. *Hordeum brachyantherum* 4*x* accession JIC line 2 had only a single fragment (11 kb), but another accession of this cytotype (H2420, lane 17) showed a profile representative of tetraploid species. In hexaploid species (lanes 18–21), all except *H. arizonicum* (lane 18) showed a profile similar to that of representative tetraploid species, but the 12–10 kb and 9·5–7·5 kb fragment classes consisted of complicated multiple bands with size and intensity variation among species. In *H. arizonicum*, faint double fragments of a new class range (4–3 kb) were detected.

For reference, representative I-genome diploid species were examined for rDNA–RFLP (Fig. 3B, Table 1). Southern analyses showed clear differentiation between Asian and American diploid species, supporting our previous FISH results (Taketa *et al*., 2001). All three Asian diploid species, *H. brevisubulatum* 2*x*, *H. bogdanii* and *H. roshevitzii* (lanes 8–10) had only a single fragment of 12–10 kb. In contrast, all American diploid species had fragments at two size classes: 9·5–7·5 kb and 2·5 kb. Among American species, *H. pusillum* (lane 13) was unique in having extra double fragments in the 3–4 kb class.

By comparing the RFLP patterns of diploids with those of polyploids, we obtained interesting findings. The 12–10 kb fragment, which is specific to Asian diploids, was detected in all American polyploid species except for *H. depressum*. The 2·5 kb fragment was conserved across all American species except for one *H. brachyantherum* 4*x* accession, but was absent in all Asian diploids. The fragments in the 9·5–7·5 kb class were highly polymorphic both between and within American species. For example, all three accessions of *H. brachyantherum* ssp. *californicum* differed in fragment patterns of this class (lanes 11, 12 and 31).

DISCUSSION

Ancestry of the perennial tetraploid species

I-genome polyploid species have not previously been analysed for their rDNA FISH patterns to any significant extent. Only two species/cytotypes, *H. brevisubulatum* 4*x* and *H. brachyantherum* 4*x*, have been described so far (Taketa *et al*., 1999*b*). Our previous FISH studies on American I-genome diploid species revealed a low level of polymorphism (Taketa *et al*., 1999*b*, 2001). Therefore, in the present study we employed both FISH and RFLP techniques for more sensitive detection of rDNA polymorphisms in the American I-genome polyploid species. Previous rDNA–RFLP analyses on *Hordeum* (Molnar and Fedak 1989;

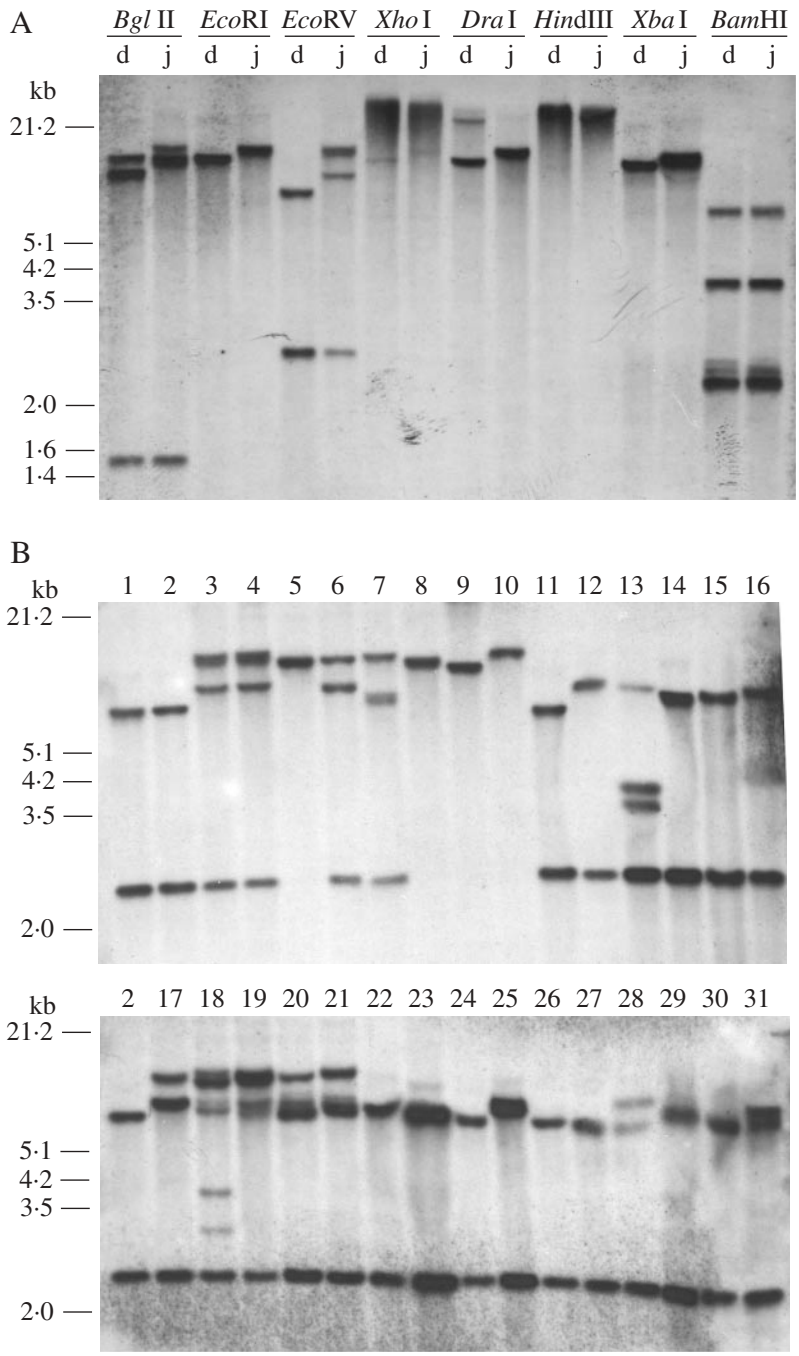

FIG. 3. Luminographs showing Southern hybridization of labelled pTa71 probe to digests of genomic DNA from (A) *H. depressum* H2089 and *H. jubatum* H1922, and (B) to *Eco*RV digests of genomic DNA from various I-genome *Hordeum* species. In (A) eight restriction enzymes were used, and 'd' and 'j' stand for DNAs of *H. depressum* and *H. jubatum*, respectively. In (B) species/accession names are indicated with numbers, which correspond to those given in Table 1.

Molnar *et al*., 1989, 1992) failed to reveal a detailed relationship among I-genome species owing to a low level of polymorphisms. However, the present study revealed that the restriction enzyme *Eco*RV is very informative in rDNA–RFLP analysis of *Hordeum*.

The similar FISH pattern in the five perennial tetraploid species (the 'jubatum type') suggests their close relationships and common origin. Because the FISH pattern of the 'jubatum type' deviates significantly from a set of four homologous chromosomes, these species are considered alloploids. Similar RFLP profiles were observed in all five perennial tetraploid species except for one *H. brachyantherum* 4*x* accession. The presence of both the 'sr SAT chromosome' pair and the Asian species-specific rDNA fragment in all 'jubatum-type' species indicates that *H. roshevitzii* is one of their diploid ancestors. The

other diploid ancestor is probably a North American 'chilense-type' species because the summed FISH signals of *H. roshevitzii* and the 'chilense type' match those of the 'jubatum type'. If we consider the current geographical distribution of the *Hordeum* species (von Bothmer *et al*., 1995), it is reasonable to assume that the 'jubatum-type' species were probably formed somewhere in Asia or North America, in which the supposed ancestral diploid species coexisted. Because *H. roshevitzii*-like diploid species are absent in Central and South America, tetraploid species in these areas are probably descendents of North American tetraploids that migrated southward.

The 'chilense-type' diploid ancestor of respective 'jubatum-type' species may be inferred from FISH and RFLP patterns. *Hordeum jubatum*, *H. guatemalense* and the standard accession of *H. brachyantherum* 4*x* seem to have an ancestor identical to *H. brachyantherum* ssp. *californicum* accession H1954, owing to the common RFLP fragments. *Hordeum brachyantherum* ssp. *californicum* includes two types of karyotypes, namely those having one or two pairs of SAT chromosomes (Linde-Laursen *et al*., 1986, 1995; Taketa *et al*., 2001). The accession H1954 carries two SAT chromosome pairs and shows the 'chilense-type' FISH pattern (Taketa *et al*., 2001); therefore this accession could be an ancestor of the three North American tetraploid species. On the basis of karyotypes, Linde-Laursen *et al*. (1995) reached a similar conclusion. Studies on chloroplast DNA also suggested that *H. brachyantherum* ssp. *californicum* is the maternal parent of these three tetraploid species (Nishikawa *et al*., 2002). On the other hand, a South American tetraploid, *H. fuegianum*, has a slightly different RFLP profile in the 9·5–7·5 kb fragment class. This may be due to a mutation or an introgression from South American diploid species. On the basis of C-banding pattern, Linde-Laursen *et al*. (1990) proposed an introgression into South American tetraploids from a South American diploid, *H. patagonicum*. We could not verify this from the RFLP pattern.

As mentioned above, *H. brachyantherum* 4*x* included a novel accession that has only a single 11-kb rDNA fragment similar to that found in Asian diploids. We suppose that this accession was probably derived from hybridization between *H. roshevitzii* and a *H. brachyantherum* ssp. *californicum* accession having an RFLP profile identical to that of *H. roshevitzii*. Such a special *H. brachyantherum* ssp. *californicum* accession has not been identified yet and might have become extinct by now. But, if present, it may represent a transitional form from Asian to American diploid species. On the basis of MI chromosome pairing, von Bothmer *et al*. (1986) concluded that, of the three North American diploids, the genome of *H. brachyantherum* ssp. *californicum* is very similar to that of the Asian diploid *H. roshevitzii*, but that the genomes of the two annuals, *H. interecedens* and *H. pusillum*, are very similar to those of the South American diploids. This may support derivation of *H. brachyantherum* ssp. *californicum* from *H. roshevitzii*. The presence of two distinct forms in *H. brachyantherum* 4*x* that differ in rDNA–RFLP profile may suggest multiple origins of this cytotype. Wide morphological variation of *H. brachyantherum* 4*x* has been reported (von Bothmer *et al*., 1993).

Ancestry of H. depressum

The FISH and RFLP patterns of the North American annual species *H. depressum* were clearly different from those of the 'jubatum-type' species. The absence of the 'sr SAT chromosome' pair in *H. depressum* may be explained by the deletion of the 18S–25S rDNA site in the 'jubatum-type', but the unique rDNA–RFLP profile of this species rules out this possibility. The absence of the Asian diploid species-specific rDNA fragment in *H. depressum* indicates that this species originated in North America independently of the 'jubatum-type' species. The nature of polyploidy in *H. depressum* has been a matter of debate. An autoploid origin of this species was suggested from a high autosyndetic pairing between the two *H. depressum* genomes in intergeneric hybrids (Sakamoto, 1974; Petersen, 1991). On the other hand, *H. depressum* was proposed to be an alloploid involving the diploid *H. brachyantherum* ssp. *californicum* as one parent and either *H. interecedens* (Baum and Bailey, 1988) or *H. pusillum* (Covas, 1949) as the other. On the basis of crossing experiments, Salomon and von Bothmer (1998) concluded that *H. depressum* arose from hybridization between *H. intercendens* and *H. brachyantherum* ssp. *californicum*. Studies on the chloroplast DNA suggested *H. brachyantherum* ssp. *californicum* as the maternal parent of *H. depressum* (Doebley *et al*., 1992; Nishikawa *et al*., 2002). The present study indicates that *H. depressum* is not a simple autoploid because its FISH pattern slightly deviates from a set of four homologous chromosomes. *Hordeum brachyantherum* ssp. *californicum*, *H. pusillum* and *H. intercedens* had a FISH pattern that is expected for a diploid ancestor of *H. depressum* (see Taketa *et al*., 2001). However, the RFLP analysis revealed that only one accession (H3317) of *H. brachyantherum* ssp. *californicum* showed a RFLP profile identical to that of *H. depressum*, and that the other species/accessions had fragment(s) not found in *H. depressum* and therefore were unsuitable as diploid ancestors. Thus, available results suggest that *H. depressum* is a segmental alloploid having two closely related genomes, one of which was derived from a specific accession of *H. brachyantherum* ssp. *californicum*. A study of repetitive DNA sequences (Svitashev *et al*., 1994) also indicated a close relationship between these two taxa. The donor of the second genome of *H. depressum* remains unknown. As suggested by Linde-Laursen *et al*. (1995), the second genome could have been derived from another accession of *H. brachyantherum* ssp. *californicum*. *Hordeum depressum* is assumed to have a strong diplodizing mechanism because it shows exclusive bivalent formation at meiosis and good seed fertility (von Bothmer *et al*., 1987). Such a mechanism ensures stable seed propagation and must have been a prerequisite for its successful establishment as an annual (Knutsson and von Bothmer, 1993). Further detailed studies on intra- and inter-specific variation on North American species are required to elucidate the ancestry of *H. depressum*.

Hexaploid species

In hexaploid species, only *H. arizonicum* is an annual/biannual and is distributed in North America, while the

other three are all perennials and grow in South America. The RFLP analysis unequivocally shows that a North American annual, *H. pusillum*, is the diploid ancestor of *H. arizonicum*. The tetraploid ancestor is probably *H. jubatum*. On the basis of crossing experiments, Rajhathy and Symko (1966) concluded that *H. arizonicum* is an amphiploid between *H. pusillum* (female parent) and *H. jubatum*. Nishikawa *et al*. (2002) reported that *H. arizonicum* and *H. pusillum* have very similar chloroplast sequences. The present results provide the first firm evidence from nuclear genomes for the involvement of *H. pusillum* in the ancestry of *H. arizonicum*. Among diploid *Hordeum* species, *H. pusillum* is known to have shorter chromosomes and smaller DNA content (Kankanpää *et al*., 1996). Two short chromosome pairs with rDNA sites in *H. arizonicum* (marked s3 and m2 in Fig. 1E) were probably derived from *H. pusillum*.

A South American hexaploid species, *H. lechleri*, has a FISH pattern very similar to that of *H. arizonicum*, but the RFLP data indicate their different origins. The present data and morphological observation (von Bothmer *et al*., 1995) suggest that the tetraploid ancestor of *H. lechleri* is probably *H. jubatum*. The diploid ancestor could not be identified because among the South American diploids there were no species matching in the FISH or RFLP pattern of rDNA.

On the basis of C-banding pattern and morphology of marker SAT chromosomes, Linde-Laursen *et al*. (1990) proposed that *H. parodii* is an alloploid between *H. tetraploidum* and *H. muticum*, and that *H. procerum* is an alloploid between *H. jubatum* and *H. cordobense*. The FISH patterns indicate that *H. parodii* and *H. procerum* have a 'jubatum-type' species, probably *H. fuegianum* or *H. tetraploidum*, as the tetraploid ancestor. It is impossible to infer their diploid ancestor based solely on the FISH pattern owing to a lack of diagnostic polymorphism (Taketa *et al*., 2001; present study). In the RFLP profile of these species, different rDNA fragments in the 9·5–7·5 kb class were intensified; *H. parodii* had a strong 7·5 kb fragment, while *H. procerum* had a strong 8·5 kb fragment. The RFLP data suggest that the diploid ancestors of *H. parodii* and *H. procerum* may be a *H. patagonicum* subspecies with the 7·5 kb fragment and *H. cordobense*, respectively.

rDNA evolution in I-genome polyploid species

In I-genome *Hordeum* species, FISH patterns of both 5S and 18S–25S rDNA sites allowed estimation of lower-ploidy-level ancestors of most polyploid species. This is because rDNA sites are highly conserved between polyploid species and their putative ancestors, generally showing additive relationships. Although the FISH signal intensities differed between corresponding rDNA sites of polyploids and their estimated ancestors, such discrepancies are probably caused by nucleolar dominance (Cermeno and Lacadena, 1985) and amplification or reduction of rDNA repeat unit sequences (Heslop-Harrison, 2000). Some I-genome polyploid species had one or two minor 18S–25S rDNA sites that were not observed in their putative ancestors. These extra minor sites were probably formed by dispersion of rDNA sites, as proposed by Dubcovsky and Dvorák (1995). This highly conserved nature of both 5S and 18S–25S rDNA sites during polyploid evolution in I-genome *Hordeum* species sharply contrasts with observations made in other plant species. In *Gossypium*, the cultivated tetraploid species *G. hirstum* had as many as four extra minor 18S–28S rDNA loci that were not found in either of the two diploid ancestral species, while 5S rDNA sites were the sum of its supposed ancestors in both number and location. In polyploid series of the *Brachyscome lineariloba* complex, 5S rDNA sites progressively increased as the ploidy level elevated, but 18S–26S rDNA was restricted to a single major locus (Adachi *et al*., 1997). In polyploids of *Sanguisorba*, however, the reverse tendency was reported (Mishima *et al*., 2002). Thus, during polyploid evolution, plant species differ in the degree of the stability of rDNA sites, and different species show different trends in rDNA site-number change.

rDNA–RFLP analysis detected rDNA polymorphisms more sensitively and corroborated the estimation of ancestry based on the FISH pattern. RFLP analysis showed that I-genome polyploid species of *Hordeum* generally retain variants of 18S–25S rDNA repeat sequences contributed by their putative ancestral species, although quantitative changes in their copy numbers after polyploidization were apparent in some species. A completely different situation was reported in *Gossypium*, where all rDNA repeats in allopolyploids were converted to one of the ancestral diploid types by concerted evolution (Wendel *et al*., 1995). In I-genome *Hordeum* species, intra- and inter-locus concerted evolution of rDNA sequences appears to be operating at a much slower rate than that reported in *Gossypium* (Wendel *et al*., 1995). The variants of 18S–25S rDNA repeat units detected by *Eco*RV digestion in I-genome *Hordeum* species probably involve changes in intergenic spacer regions, but this must be confirmed by constructing restriction site maps. Moreover, chromosomal distribution of rDNA repeat unit variants needs to be clarified. Phylogenic studies of Triticeae species have utilized 5S rDNA spacer sequences (Baum and Johnson, 1998) and internal transcribed spacer (ITS) sequences of 18S–25S rDNA (Hsiao *et al*., 1995) with some success. However, application of these parameters to the phylogenetic analysis of I-genome polyploid *Hordeum* species may require some caution because the materials may violate the basic assumption of homogeneity between the repeated units of rDNA. Due to the complications associated with polyploidy, only a few polyploid *Hordeum* species have been analysed by other molecular markers and comparative sequencing approaches, mostly failing to reveal their ancestors (Komatsuda *et al*., 2001; de Bustos *et al*., 2002; El-Rabey *et al*., 2002). In conclusion, the highly conserved nature of rDNA sites during polyploid evolution in the I-genome group of *Hordeum* has enabled the estimation of the ancestry of polyploid species based on the FISH and RFLP data. The genus *Hordeum* is an excellent material to study the patterns of plant evolution because of availability of many species and accessions with a variety of ploidy levels, life forms, reproduction modes and adaptability. Large amounts of rDNA FISH and cytological data have been accumulated in this genus (Leitch and Heslop-Harrison, 1992, 1993; Pedersen and Linde-Laursen, 1994; Linde-Laursen *et al*., 1995; de Bustos *et al*., 1996; Taketa

et al., 1999*b*, 2001, 2003; present study). Future elucidation of homoeology of wild barley chromosomes would allow comparative analysis of karyotype evolution not only within the genus but also with other genera in the grass family. Moreover, such analysis may help solve an important question: why polyploid series were developed only in wild *Hordeum* species, but not in cultivated barley (*H. vulgare* ssp. *vulgare*, $2n = 2n = 14$), an important cereal crop.

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APPENDIX

Collection sites of Hordeum *species with the I genome analysed in this study*

Species	Ploidy	Accession	Collection site
FISH			
H. jubatum L.	4*x*	H1922	USA: New Mexico, E Santa Fe
	4*x*	H1976	USA: California, Solano co,
H. guatemalense Both.& al.	4*x*	H2299	Guatemala: dep. Huehuetenango
H. depressum (Scribn.&Sm.) Rydb.	4*x*	H2089	USA: California, San Joaquin co.
	4*x*	H2005	USA: California, San Luis Obispo co.
H. fuegianum Both.& al.	4*x*	H1418	Argentina: Tierra del Fuego
H. tetraploidum Covas	4*x*	H6198	Argentina: Santa Cruz, Rio Pelke
H. arizonicum Cov.	6*x*	H3254	USA: Arizona, Cochise co.
H. lechleri (Steud.) Schenck	6*x*	H6104	Argentina: Tierra del Fuego
H. parodii Covas	6*x*	H6294	Argentina: prov. Chubut
H. procerum Nevski	6*x*	H1789	Argentina: prov. San Juan
H. comosum Presl	2*x*	H10608	Argentina
Species only used for Southern hybridizaiton			
H. brevisubulatum (Trin.) Link			
ssp. *violaceum* (Boiss. & Hohen.) Tzvel	2*x*	H315	Iran: prov. Mazanderan
H. bogdanii Wil.	2*x*	H4014	Pakistan: Gilgit, Nagar valley
H. roshevitzii Bowden	2*x*	H9152	China: Gansu
H. brachyantherum Nevski			
ssp. *californicum* (Cov. & Steb.) Both. & et al.	2*x*	H3317	USA: California, Ventura co
	2*x*	H1954	USA: California, Carmel vy, Haystack Hill
	2*x*	H2419	USA: California, San Luis Obispo co
ssp. *brachyantherum*	4*x*	JIC line2	Unknown
	4*x*	H2420	USA: California, San Luis Obispo co
H. pusillum Nutt.	2*x*	H722	USA: Texas, Childress co
H. intercedens Nevski	2*x*	H2310	USA: California, Ventura co
H. chilense Roem. & Schult.	2*x*	JIC line 1	Unknown
H. muticum Presl	2*x*	H6479	Argentina: prov. Jujuy
H. cordobense Both. & al.	2*x*	H6429	Argentina: prov. Mendoza
H. flexuosum Steud.	2*x*	H1116	Argentina: prov. Buenos Aires
H. patagonicum (Haum.) Cov.			
ssp. *musterisii* (Nico.) Both. & al.	2*x*	H1358	Argentina: prov. Santa Cruz
ssp. *patagonicum*	2*x*	H6052	Argentina: prov. Santa Cruz
ssp. *setifolium* (Paro. & Nico.) Both. & al.	2*x*	H1366	Argentina: prov. Santa Cruz
ssp. *santacrucense* (Paro. & Nico.) Both. & al.	2*x*	H1353	Argentina: prov. Santa Cruz
H. pubiflorum Hook. f.			
ssp. *halophilum* Grise.	2*x*	H1348	Argentina: prov. Santa Cruz
ssp. *pubiflorum* Hook. f.	2*x*	H1296	Argentina: prov. Santa Cruz
H. stenostachys Godr.	2*x*	H1108	Argentina: prov. Buenos Aires

Seeds of these accessions are preserved at Kagawa University, Japan and Nordic Gene Bank, Sweden.